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Using immunohistochemistry that employed direct conjugation of alkaline phosphatase to a monoclonal antibody (PF 2A10) to the circumsporozoite protein of Plasmodium falciparum, the appearance of the CS protein was followed over time in the mosquito. The CS protein first appeared in vacuolated oocysts and as oocysts matured to the sporoblast, the amount of CS protein increased. CS protein was evident on the surface of sporozoites released into the hemolymph from ruptured oocysts between days 10-13 after the infective blood meal. Sporozoites did not remain very long in the hemolymph. Between 11-15 days after the infective blood meal, sporozoites appeared on the surfaces of the salivary glands depositing a significant amount of CS protein on the glands as they invaded. Small immunogenic pits were observed which mark the entrance of the parasite into the gland. here was large variability in the labelling of parasites once they were in the glands.

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Original Article

The Distribution of Circumsporozoite Protein (CS) in *Anopheles stephensi* Mosquitoes Infected with *Plasmodium falciparum* Malaria[1]

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We monitored the distribution of *Plasmodium falciparum* circumsporozoite protein (CS) in *Anopheles stephensi* using an immunohistochemical method. An alkaline phosphatase-labeled monoclonal antibody, specific for the CS protein of *P. falciparum*, was incubated with tissue sections from infected and non-infected mosquitoes. Sections were stained for phosphatase activity using a new fuchsin/naphthol AS-BI phosphate capture system. Distribution of the CS protein in mosquitoes was dependent on the time after post-infective blood meal. CS protein was first detected in immature oocysts on the mosquito midgut. As oocysts differentiated to mature sporoblasts, detectable CS protein increased. Between 11-16 days post infective blood meal, CS protein was detected on the surface of sporozoites that were released into the hemolymph from oocysts. Although sporozoites were found throughout the hemocoel, they were most frequently associated with the salivary glands and flight muscle. Once in the salivary glands, sporozoites massed into bundles. The amount of CS protein associated with bundles of sporozoites was highly variable. (*J Histochem Cytochem 38:475–481, 1990*)

KEY WORDS: *Plasmodium falciparum*; *Anopheles stephensi*; Circumsporozoite protein; Fuchsin/naphthol AS-BI phosphate capture system.

Introduction

The human malarial parasite *Plasmodium falciparum* undergoes fertilization and sporogony in the mosquito. During a blood meal, the mosquito ingests the male and female gametocytes from the malaria-infected human host. Fertilization occurs in the midgut of the mosquito, producing a zygote which rapidly differentiates to an elongated motile ookinete. The *P. falciparum* ookinete traverses the midgut wall by an intercellular route between epithelial cells (Meis and Ponnudurai, 1987). Beneath the basement membrane the ookinete rounds up, forming an oocyst which quickly undergoes reduction division. The oocyst differentiates, producing sporozoites which invade the salivary glands and can be transmitted to a human host via a mosquito bite.

The surface of the sporozoite is covered by the circumsporozoite (CS) protein, the primary antigen used in the development of malarial sporozoite vaccines (Ballou et al., 1987). The CS protein of several *Plasmodium* species has been detected in maturing oocysts (Boulanger et al., 1988; Hamilton et al., 1988; Nagasawa et al., 1988), and sporozoites from oocysts and in salivary glands (Boulanger et al., 1988; Nagasawa et al., 1988), using immunogold electron microscopy and an indirect fluorescent antibody test (IFAT). These studies demonstrated the synthesis of CS proteins in oocysts and localized the CS protein on the surface of the sporozoite, but provided no information on the distribution of the CS protein in whole mosquitoes or on the migration route of sporozoites from oocysts to the salivary glands. Recently, an enzyme-linked immunosorbent assay (ELISA) was used to study the distribution of CS protein in mosquito tissues (Robert et al., 1988).

Using an immunohistochemical approach, we examined whole mosquito serial sections rather than selected tissues in an attempt to visualize the distribution of CS protein and/or parasites in mosquitoes.

Materials and Methods

Three- to 5-day-old female *Anopheles stephensi* mosquitoes were infected by feeding them cultured *Plasmodium falciparum* (NF 54) gametocytes suspended in defibrinated human blood (Rutledge et al., 1964). Eight to 22 days after the infective blood meal, groups of six to nine mosquitoes were fixed in buffered formalin. Legs and wings were removed and the thoracic and abdominal cuticle was punctured with an insect pin to ensure penetration of the formalin. Mosquitoes (two or three per block) were embedded in paraffin and longitudinal serial sections were cut (6 µm; c. 90–100 sections per mosquito).

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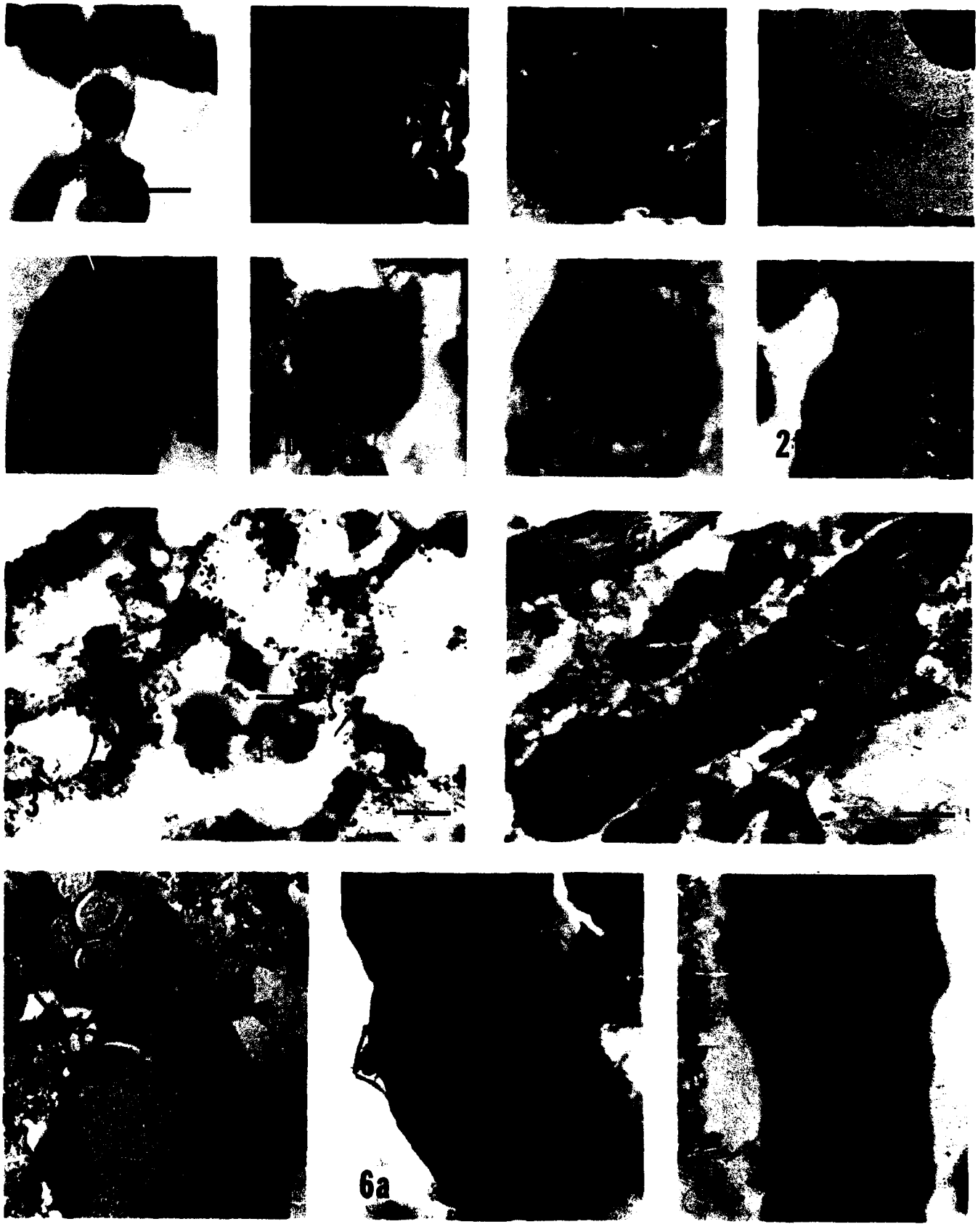
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Sections were deparaffinized in Hemo-De (twice for 5 min), hydrated for 2 min each through graded alcohols, and rinsed in tap water. Endogenous alkaline phosphatase activity was eliminated by a 5-min incubation in Bouin's reagent, followed by washes in tap water (three times for 3 min) and PBS (pH 7.2, three times for 3 min). Slides were then incubated in blocking buffer (1% bovine serum albumin, 0.5% casein, and 0.01% thimerasol in PBS, 30 min), followed by alkaline phosphatase-labeled 2A10 monoclonal antibody (1 μg MAb/ml, 1 hr). MAb 2A10 recognizes an immunodominant epitope on the CS protein of *P. falciparum* (Zavala et al., 1983). Slides were then washed in PBS (three times for 3 min), and alkaline phosphatase activity was demonstrated using naphthol AS-BI phosphate and hexazotized new fuchsin (Histomark RED; Kirkegaard Perry Laboratories, Gaithersburg, MD). A pink to red color develops at sites of enzyme activity. The reaction was stopped by washing slides in tap water (5 min) before counterstaining with hematoxylin (15–20 sec), and blueing in ammonia water (0.002%). Slides were rinsed in tap water (2 min), dehydrated through graded alcohols, cleared in Hemo-De (twice for 3 min), and mounted.

All serial sections were examined for the presence of CS protein, and the number of infected mosquitoes was determined. The stage of the malarial infection in mosquitoes is given as days post infective blood meal. Selected sections were photographed using 35-mm Kodacolor 200 ASA film and a Vanox-S Olympus microscope.

Results

Microscopic examination of immunologically stained whole mosquito serial sections demonstrated the presence of CS protein 8–22 days after the infective blood meal. The tissue distribution of CS protein was dependent on the time after the infective feed that mosquitoes were fixed and on the level of infection. Although 78% of mosquitoes (7/9) fixed at Day 8 contained oocysts, CS protein was detected in only 29% of the infected individuals (2/7). In these mosquitoes, CS protein was demonstrated only in oocysts. In two small oocysts (approximately 15 μm in diameter), the CS protein was associated with peripheral membranes and cytoplasmic vesicles (Figure 1a). Most oocysts observed at this time were less than 15 μm in diameter and contained granular pigment (Figure 1b).

Oocysts positive for CS protein and in various stages of development were observed in the midgut walls of mosquitoes fixed at Days 10 and 11, at which time 83% of the mosquitoes (15/18) were infected. Oocysts were observed up to Day 16; thereafter, the only sign of midgut infection was the presence of intensely stained membranes on the midgut (described below). Invaginations of the plasmalemma from the oocyst wall demonstrated the presence of CS protein (Figure 2a). This was most commonly observed in mosquitoes fixed at Day 10. In oocysts characterized by cytoplasmic fragmentation, CS protein was associated with membranous vesicles located among islands of cytoplasm (Figure 2b). CS protein synthesis increased, as indicated by an increase in red-staining membranes which now formed large clefts among the fragmented cytoplasm (Figure 2c). Initially, immature sporozoites did not express CS protein; however, as the sporoblast differentiated, bundles of mature sporozoites immunoreactive for CS protein were observed (Figure 2d). Sporozoites released into the hemocoel from mature ruptured oocysts also demonstrated CS protein immunoreactivity (Figure 2e), as did remnant oocyst membranes left in the gut wall (Figure 2f).

Infected mosquitoes fixed at Day 10 revealed CS protein only in differentiating oocysts. By day 11, 22% of the infected mosquitoes (2/9) also demonstrated CS protein on the surface of sporozoites in the hemolymph, and on several tissue types. Sporozoites in the abdominal hemolymph were easiest to detect proximal to ruptured oocysts. In the thorax they were observed near salivary glands. The surface of the parasite was pink to bright red, as contrasted with the purple-blue of the hemolymph (Figure 3). Sporozoites were also observed throughout the hemocoel (except in wings and legs, which were removed), often associated with muscle and tracheal tissues rather than with circulating hemolymph. In the abdomen of mosquitoes fixed between 11–16 days, sporozoites adhered to mid- and hindgut muscles, alary muscles, the peritoneal membrane lining the ovaries, the large tracheal branch passing near the rectum, the elastic lining of the crop, and the malpighian tubules. This adhesion was not as great as that observed between sporozoites and the salivary glands. After Day 16 few sporozoites were detected in the abdomen.

Between Days 11–16 the surfaces of the salivary glands were positive for CS protein (Figure 4). CS protein was detected on the surface of the salivary glands in 22% (2/9) of infected mosquitoes fixed at Day 11. By Day 12, 50% of infected mosquitoes (3/6) contained CS protein associated with the salivary glands, and by Days 14–16 CS protein was localized on/in the salivary glands of all infected mosquitoes (12/15). During this time it was difficult to determine if CS protein was always associated with the sporozoite or was deposited on the gland by the sporozoite. Figure 5 illustrates sporozoites adhering to the distal region of a salivary gland lateral lobe (Arrow A). Arrow B defines a more proximal region of the lobe in which the CS protein was not associated with the

Figure 1. First appearance of circumsporozoite (CS) protein at Day 8 in midgut of mosquitoes. (a) Small oocyst in which CS protein was localized in peripheral membranes; (b) undifferentiated pigmented oocysts from same mosquito. Bar = 15 μm.

Figure 2. Stages of oocyst differentiation: (a–d) Day 10; (e–f) Day 12. (a) Periperal vacuolization of oocyst in which CS protein was detected on vacuole membranes. (b) Fragmentation of oocyst cytoplasm; CS protein present within cytoplasmic islands. (c) Increased production of CS protein in cytoplasm. (d) Mature sporoblast containing mature sporozoites. (e) Release of sporozoites from oocyst. (f) Immunoreactive membrane left in midgut wall after rupture of oocyst. Bar = 20 μm.

Figure 3. Localization of CS protein on surface of hemolymph sporozoites on Day 10. Bar = 15 μm.

Figure 4. Detection of CS protein on surfaces of salivary glands from mosquito fixed on Day 14. Bar = 30 μm.

Figure 5. Distal region of salivary gland lateral lobe in which CS protein was detected on sporozoites on gland (arrow A) and more proximal region where CS protein appeared to coat gland (arrow B). Mosquitoes fixed on Day 14. Bar = 15 μm.

Figure 6. Small immunoreactive regions (arrows) localized on gland surface in mosquitoes fixed on Days 12–14. (a) Distal region of medial lobe; (b) distal region of lateral lobe. Bar = 15 μm.


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8a


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parasite. Small circular immunoreactive areas (1–2 μm in diameter) were localized on the surface of the gland. These were confined to the medial lobe (Figure 6a) and the distal portion of the lateral lobes (Figure 6b). Although they were observed repeatedly in the glands of mosquitoes fixed between Days 11–16, they were observed only in lightly infected mosquitoes. The large numbers of sporozoites associated with massive infection probably obscured the presence of these regions. In longitudinal sections from the distal portion of the medial salivary gland lobe, CS protein was localized either between cells or in the scant cell cytoplasm (Figure 7). Limits of light microscopy magnification prevented such a determination. After Day 18 the surfaces of the salivary glands were less immunoreactive for CS protein; however, the cells and ducts of the glands were often reactive.

Whereas the CS protein was readily detected on the surface of sporozoites outside the salivary gland, it was difficult to detect the CS protein on sporozoites that resided in glandular secretions. Sporozoites usually appeared as distinct groups in both salivary gland cells and ducts. Bundles of sporozoites varied greatly with respect to CS protein immunoreactivity. High, moderate, low, or no association with CS protein was observed (Figures 8a–8d). In mosquitoes fixed at Day 18, 57% of the serial sections containing salivary gland demonstrated moderate to high levels of sporozoite CS protein, whereas the remainder did not. Similar results were obtained from mosquitoes fixed at Day 21. As shown in Figure 8, sporozoites residing in the distal lateral lobe region of salivary glands were most frequently localized in the ducts; however, in the more proximal regions bundles of sporozoites were found in cells surrounding the lumen (Figure 9). Frequently, sporozoites were detected in distal duct vesicles of the glandular lateral lobes. Within these vesicles sporozoites appeared as bundles, with the CS protein most clearly associated with the vesicle periphery rather than with the parasites (Figure 10).

Sporozoites also showed an affinity for muscle tissue. Where large numbers of sporozoites were present (Figure 11), the surfaces of insect flight muscles demonstrated high CS protein immunoreactivity (Figure 12). Sporozoites were also found associated with leg muscles on the coxae.

Discussion

The new fuchsin/naphthol AS-BI phosphate method was used to demonstrate the synthesis of the CS protein during oocyst differentiation to the mature sporoblast. The appearance of CS protein and its localization in oocyst membranes paralleled the report of Terzakis et al. (1967), in which they described the appearance of small tufts of material in maturing *P. gallinaceum* oocysts. These first completely line capsular invaginations and later the clefts in differentiating oocysts. Similarly, Nagasawa et al. (1988) and Posthuma et al. (1988), using immunogold electron microscopy, showed that the CS protein of *P. malariae* and *P. falciparum*, respectively, was synthesized in immature oocysts before sporozoite formation. In this study, we further demonstrate the presence of CS protein with remnant oocyst membranes after release of sporozoites.

After oocysts released sporozoites into the hemolymph (Days 11–14), the distribution of CS protein indicated that parasites did not adhere to most tissues, except for the salivary glands and thoracic muscles. Using an ELISA, Robert et al. (1988) localized the CS protein of *P. falciparum* primarily in the salivary glands and thorax of infected *An. gambaie* dissected at Day 14.

The migration of sporozoites from oocysts to salivary glands could be active, passive, or both. If active, the sporozoite would use a gliding motility to reach the gland. Sporozoites that move over a substratum in vitro leave behind trails of CS protein (Stewart and Vanderberg, 1988). No free CS protein was detected in the hemolymph, on hemocytes, or near masses of sporozoites in the hemolymph. Sporozoites were found throughout the hemocoel but were present in particularly high concentrations in the thorax, suggesting transport during the normal course of hemolymph circulation.

Oelerich (1967) suggested the existence of a positive organotropism, i.e., an attraction of the sporozoites by the salivary glands. The congregation of sporozoites in the vicinity of salivary glands before glandular invasion (Figure 4) supports a taxic mechanism. Several polysaccharides have been identified that orient protozoa towards or away from a stimulus (Van Houten, 1988). Mucopolysaccharides and carbohydrate–protein complexes have been demonstrated in the salivary glands of *Aedes aegypti* mosquitoes (Orr et al., 1961).

King (1988) proposed that sporozoites invade salivary glands using a mechanism similar to invasion of erythrocytes by the merozoite stage of the parasite. Invasion of mosquito salivary glands by plasmodia has not been reported. In *Lutzomya vexator* (Diptera: Psychodidae), the anterior of *P. mexicanum* sporozoites made contact with the salivary gland cell at a 90° angle, causing an invagination of the salivary gland membrane (Klein et al., 1988). The immunoreactive areas localized on the surface of the lateral and medial lobes (Figures 6a and 6b) may mark the entry path of motile sporozoites into the glands. Ramsey et al. (1981) showed that

Figure 7. CS protein localized (arrow) in region between cells of the medial lobe in mosquitoes fixed on Day 14. Bar = 15 μm.

Figure 8. Variation of CS protein in lateral lobes of salivary glands. (a) Highly immunoreactive duct in lateral lobe from mosquito fixed on Day 21. (b) Moderate association of CS protein with bundles of sporozoites in mosquito fixed at Day 18. (c) Little CS protein associated with parasites in mosquito fixed on Day 18. (d) No association of CS protein with sporozoites in mosquito fixed on Day 21. Bar = 15 μm.

Figure 9. Bundles of sporozoites in proximal region of lateral lobe of gland in which parasites were located both in the duct and in cells surrounding duct on Day 18. Bar = 15 μm.

Figure 10. Cross-section of distal region of lateral lobe in which CS protein was localized on peripheral membrane that surrounded parasites on Day 18. Bar = 15 μm.

Figure 11. CS protein on surface of parasites located in hemolymph near flight muscles on Day 14. Bar = 15 μm.

Figure 12. CS protein and/or sporozoites detected on the surface of mosquito flight muscle on day 14. Bar = 15 μm.

sporozoite surface antigens appeared associated with the target membrane during entry into W138 cells, and membrane-limited vacuoles beneath the plasma membrane in *An. stephensi* salivary gland cells invaded by *P. berghei* have been described (Sterling et al., 1973). Penetration of the salivary glands by parasites could also involve an intercellular route, as demonstrated for the ookinete stage of *P. falciparum* in *An. stephensi* (Meis and Ponnudurai, 1987). In the distal lateral lobes, sporozoites were repeatedly observed in the ducts of glands before appearing in the secretory cavities. In the medial lobe of the gland, CS protein was observed in the region between cells (Figure 7).

Variations in the detection of CS protein were observed only when sporozoites were located in the salivary glands (Figures 8a–8d). Sporozoites released from the gland by trituration, as well as sporozoites detected in the hemolymph or on the surface of the glands, did not demonstrate such variations. Recently, Rosario et al. (1989) demonstrated that mosquitoes fed anti-CS *P. falciparum* antibodies were neutralized by MAb 2A10 but not by vaccine sera. These authors suggested that variation in the conformational expression of the repeat region of the CS protein may have a profound impact on sporozoite interaction with anti-CS antibodies. An interaction of the CS protein with glandular secretions may hide or conformationally change the repeat region that MAb 2A10 recognizes, so that detection of the CS protein would be difficult or variable, as observed when sporozoites were in the salivary glands. Hidden antigens have been described for secretory IgA where an association between the J chain and a secretory glycoprotein makes it almost undetectable by immunohistochemistry (Brandtzaeg, 1982). It has been suggested that the association of IgA with glycoprotein prevents proteolytic attack by enzymes and facilitates its transport into secretions (Roitt et al., 1985). Likewise, an association between sporozoites and glandular secretions could protect the parasite and facilitate transport out of the gland during feeding on the host.

Erythrocytes infected with *P. falciparum* contain cytoplasmic clefts and vesicles that originate from the parasitophorous vacuole membrane and contain a common parasite antigen (Kara et al., 1988). Similarly, the peripheries of vesicular structures identified in this study were immunoreactive for a sporozoite antigen, the CS protein (Figure 10).

The occurrence of *Plasmodium* sporozoites in the hemolymph near muscle tissue, but not necessarily adhering to muscle fibers, has been described (Wenyon, 1926). *Haemoproteus columbae* sporozoites have been shown to invade longitudinal muscle cells surrounding the salivary glands of *Pseudolynchia canariensis* (Diptera: Hippoboscidae) (Klei and De Giusti, 1973). Adhesion of sporozoites to muscle tissue was evident between Days 11–21, especially in heavily infected mosquitoes; however, sporozoites were never observed in muscle fibers. Interestingly, both Schiefer et al. (1977) and Rowland and Boersma (1988) have demonstrated an impairment of flight activity in malaria-infected *An. stephensi* mosquitoes.

The immunohistochemical procedure used in processing tissue sections provided an alternative approach to the fluorescent and immunogold techniques most commonly used to detect sporozoites and CS protein. We have demonstrated the presence of CS protein in differentiating oocysts, on remnant membranes left on the midgut wall after release of sporozoites, on the surfaces of sporozoites and salivary glands, and on the periphery of vesicles surrounding groupings of sporozoites in the salivary gland. The variability in detecting the CS protein on parasites in the salivary gland may be related to an interaction between sporozoites and gland secretions.

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